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NOTE

Identification and Quantification of Carotenoids in Paprika Fruits and Cabbage, Kale, and Lettuce Leaves

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Abstract Twelve carotenoids were identified in Korean leafy vegetables and paprikas. by high-performance liquid chromatography, Carotenoid contents varied greatly, with red paprika having a higher antheraxanthin and capsanthin contents than other paprikas. Orange paprika had higher levels of zeaxanthin, β-cryptoxanthin, lutein, and α-carotene compared to those of other paprikas. The results of Pearson's correlation analysis using quantitative data of carotenoids revealed that significant positive relationships were apparent between capsanthin and antheraxanthin (r =0.9870, p <0.0001), zeaxanthin and α-cryptoxanthin (r =0.9951, p <0.0001), as well as lutein and α-carotene (r =0.9612, p <0.0001). Because the correlations between carotenoids levels have provided valuable information regarding metabolic associations, this technique will contribute to identifying metabolic links for carotenoid biosynthesis.

Keywords cabbage carotenoids · high-performance liquid chromatography · kale · lettuce · paprika

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Introduction

Carotenoids are a widely distributed group of naturally occurring pigments, usually red, orange, and yellow in color. They consist of 40 carbon molecules and are the second largest pigment group in nature, with over 650 members identified to date (Hornero-Méndez and Mínguez-Mosquera, 2000). Thus, they are used extensively as safe and natural colorants for food, feed, and cosmetics. A number of carotenoids, including α-carotene, β-carotene and β-chryptoxanthin have pro-vitamin A activity due to their conversion to retinal by mammals. The xanthophylls, lutein, and zeaxanthin are also known to provide protection against age-related macular degeneration, which is mediated by their ability to quench singlet oxygen and filter blue light in the retina (Landrum and Bone, 2001).

The carotenoids are essential components of photosynthetic organisms. Some of them such as 9-cis-epoxycarotenoids serve as precursors of abscisic acid, a key phytohormone in plant growth and development, and are involved in various stress responses (Qin and Zeevaart, 1999; Agustí et al., 2007). They also have various essential roles in the physiological processes of plants such as harvesting light for photosynthesis, protecting the photosystem from photooxidation, and stabilization of the lipid membranes (Frank and Cogdell, 1996; Havaux, 1998; Ledford and Niyogi, 2005). They therefore play a major role in fruit, root, and tuber coloration and in the nutritional quality of horticultural crops.

The extraction, separation, and identification of a wide range of compounds in a single measurement (metabolic profiling) should be a useful tool for assessing food quality. To the best of our knowledge, the carotenoid profiles of commonly consumed fruits and vegetables in Korea such as cabbage, kale, lettuce, and paprika have not been reported. The thermal instability of carotenoids excludes their separation by gas chromatography, and thus high-performance liquid chromatography (HPLC) is the most

effective and accurate tool for determining their separation and quantification (Fraser et al., 2000). The present study investigated the application of HPLC techniques to the screening of carotenoids in a selection of commonly consumed fruits and vegetables, and further characterized the carotenoids in various colored paprika fruits to determine their phytochemical diversity.

Materials and Methods

Samples and chemicals. Twenty-four carotenoids were obtained from CaroteNature (Switzerland): bixin, violaxanthin, capsorubin, antheraxanthin, capsanthin, adonixanthin, adonirubin, lutein, zeaxanthin, canthaxanthin, citranaxanthin, α-cryptoxanthin, β-cryptoxanthin, echinenone, 13*Z*-β-carotene, α-carotene, (all-*E*)-β-carotene, 9*Z*-β-carotene, astaxanthin dipalmitate, γ-carotene, lutein dipalmitate, zeaxanthin dipalmitate, lycopene, and β-apo-8'-carotenal. The paprikas were purchased from local supermarkets, and the kale, lettuce, and cabbage samples were provided by Chungnam National University (Korea). The samples were freeze-dried at −80°C for at least 72 h, after which they were ground into a fine powder using a mortar and pestle.

Extraction and analysis of carotenoids. The carotenoids were extracted and measured by HPLC as previously described (Park et al., 2013). Briefly, the carotenoids were extracted from samples (0.1 g) by adding 3 mL ethanol containing 0.1% ascorbic acid (w/v) and incubation at 85°C for 5 min in a water bath. Saponification removes the fatty acids, leaving only the free pigments, which can then be separated by HPLC to make available for identification of the different carotenoids by their functional end groups. The carotenoid extracts were therefore saponified with potassium hydroxide (120 μL, 80% w/v) at 85°C for 10 min. After saponification, the samples were placed on ice, and cold deionized water (1.5 mL) was added. Based on its carotenoid characteristics and retention time, β-Apo-8'-carotenal (0.2 mL, 25 μg/mL) was chosen as an internal standard. To separate the layers, the carotenoids were extracted twice with hexane (1.5 mL) by centrifugation at 1,200×*g* for 5 min at 4°C. The aliquots of the extracts were then dried under a stream of nitrogen and redissolved in 50:50 (v/v) dichloromethane/methanol before the HPLC analysis. The carotenoids were separated in a C30 YMC column (250×4.6 mm, 3 μm; YMC Co., Japan) by an Agilent 1100 HPLC instrument (France) equipped with a photodiode array detector, and the chromatograms were generated at 450 nm. Solvent A consisted of methanol/water (92:8 v/v) with 10 mM ammonium acetate and solvent B consisted of 100% methyl *tert*-butyl ether. The gradient elution was performed at 1 mL/min under the following conditions (%A/%B): 0 min, 90/10; 20 min; 83/17; 29 min, 75/25; 35 min, 30/70; 40 min, 30/70; 42 min, 25/75; 45 min, 90/10; and 55 min, 90/10. Identification of carotenoids was carried out by HPLC through the combined use of the retention time and co-elution with available authentic standards.

Statistical analysis. All analyses were performed at least three times. Experimental data were determined by analysis of variance, and significant differences among the means were determined by Duncan's multiple-range test. Pearson's correlation analysis was carried out among the contents of twelve carotenoids. All statistical analyses were performed using the SAS 9.2 software package (SAS Institute, USA).

Results and Discussion

Identification of carotenoids. Under the HPLC conditions described in the 'Materials and Methods', the peaks of the carotenoids were detected within 42 min, which were then used to determine the carotenoids in the samples based on the retention times of the standards (Fig. 1). The extracts from the investigated products had diverse metabolic profiles. As shown in Fig. 1, violaxanthin, lutein, zeaxanthin, 13*Z*-β-carotene, α-carotene, (all-*E*)-β-carotene, and 9*Z*-β-carotene were detected in the leaves of lettuce. Previous studies have shown that the major carotenoids in kale and lettuce leaves are lutein and β-carotene (Kopsell et al., 2007; Cruz et al., 2012), and Burns et al. (2003) identified neoxanthin, violaxanthin, lutein, and all trans-β-carotene in lettuce by HPLC.

The yellow, orange, and red colors of paprika originate from the carotenoid pigments produced in the fruit during ripening. They include the yellow pigments lutein, zeaxanthin, antheraxanthin, violaxanthin, the α- and β-cryptoxanthins, and the α- and β-carotenes; as well as the red pigments capsanthin, capsorubin, and cryptocapsin that are distinctive to the capsicums (Deli and Molnár, 2002). In the present study, 12 carotenoids were identified in the paprika fruits: violaxanthin, antheraxanthin, capsanthin, lutein, zeaxanthin, α- and β-cryptoxanthins, the 13*Z*-, α-, *E*-β-, and 9*Z*-β-carotenes, and lycopene (Fig. 1).

Quantification of carotenoids. To quantify the 12 carotenoids identified in the samples, calibration curves were drawn by plotting three concentrations of the carotenoid standards according to their peak area ratios to β-apo-8'-carotenal. The concentrations of 1–5 μg/mL carotenoids were prepared for HPLC and spectrophotometric calibration essentially as described by Howe and Tanumihardjo (2006). The stock solutions of 1 mg per 10 mL of 50:50 (v/v) dichloromethane/methanol were made and then diluted further with the same solution. The calibration curves of 12 carotenoids measured at different ranges (1–5 μg/mL) were linear with good correlation coefficients (r^2 =0.954–0.999).

The green vegetables kale and lettuce showed similar elution profiles (Table 1), and all three contained high levels of lutein and all-trans-β-carotene. The lutein content in the kale ranged from 495.20 to 552.10 μg/g dry weight (DW). Carotenoid composition can vary with variety of culture, cultivation conditions, state of maturity, post-harvest and storage handling, climate and geographical localization, sample type, and the part of the plant (Mercadante and Rodriguez-Amaya, 1990; de Azevedo and Rodriguez-Amaya, 2005). However, a previous work has shown that the lutein content in kale leaves ranges from 44.0–57.4 μg/g fresh weight

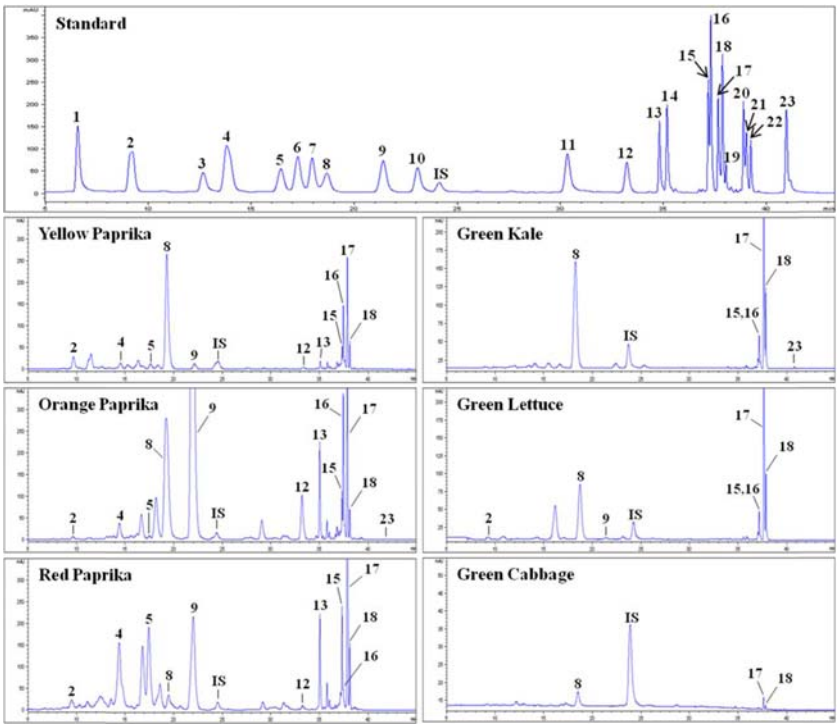

Fig. 1 HPLC chromatograms of 23 carotenoid standards and the pigments extracted from fruits and vegetables. Peaks: 1, bixin; 2, violaxanthin; 3, capsorubin; 4, antheraxanthin; 5, capsanthin; 6, adonixanthin; 7, adonirubin; 8, lutein; 9, zeaxanthin; 10, canthaxanthin; 11, citranaxanthin; 12, α-cryptoxanthin; 13, β-cryptoxanthin; 14, echinenone; 15, 13*Z*-β-carotene; 16, α-carotene; 17, *E*-β-carotene; 18, 9*Z*-β-carotene; 19, astaxanthin dipalmitate; 20, γ-carotene; 21, lutein dipalmitate; 22, zeaxanthin dipalmitate; 23, lycopene; and IS, internal standard (β-apo-8-carotenal).

(396.0–516.6 μg/g DW) (de Azevedo and Rodriguez-Amaya, 2005). Although lutein has no function as a vitamin A precursor, it is a principal component of the macular pigments in the eyes.

The composition of the carotenoid pigments produced by paprika has a long history of investigation (Deli and Molnár, 2002). A detailed pioneering work on the qualitative distribution of carotenoids in red paprika revealed that the red carotenoids such as capsanthin, capsorubin, and cryptoxanthin are formed from the 5,6-epoxy-carotenoids (Cholnoky et al., 1955). In the present study, the carotenoid content varied significantly among the red, orange, and yellow paprikas, with the levels of capsanthin and antheraxanthin in red paprika being significantly higher than those in yellow and orange paprika (Table 1). Capsanthin can be synthesized from antheraxanthin, a 5,6-epoxy-xanthophyll. Orange paprika had significantly higher levels of zeaxanthin and the α- and β-cryptoxanthins than did the other paprikas. Deli et al. (1996) also reported that orange paprika had higher zeaxanthin levels in comparison to other fruits at different stages of maturation. Furthermore, the levels of lutein and α-carotene were higher in orange paprika than in the other paprikas. Correlations between metabolites are the net result of direct enzymatic conversions and indirect cellular regulation of transcriptional or biochemical processes.

Table 1 Contents (μg/g on a dry weight basis) of carotenoids in fruits and vegetables[a]

	Viola[b]	Anthera	Capsanthin	Lutein	Zeaxanthin	α-Crypto	β-Crypto	13*Z*-β-Caro	α-Carotene	*E*-β-Caro	9*Z*-β-Caro	Lycopene	Sum
YP[c]	12.23± 1.89a	3.94± 0.73c	3.21± 0.16b	143.70± 3.98d	2.64± 0.48c	1.66± 0.15c	2.02± 0.20c	2.75± 0.21d	5.72± 0.93b	25.88± 1.10e	3.38± 0.27e	ND[d]	207.16± 5.94
OP	2.94± 0.54c	12.38± 4.16b	2.73± 0.12b	319.48± 1.05c	449.29± 5.54a	46.15± 0.96a	33.53± 0.61a	9.47± 0.40c	21.43± 1.83a	72.69± 6.22d	4.99± 0.23d	0.25± 0.07b	975.33± 10.29
RP	8.09± 0.72b	64.65± 1.35a	66.45± 1.99a	21.39± 1.17e	69.39± 7.59b	4.09± 0.27b	27.86± 1.15b	20.55± 2.94b	2.41± 0.19c	163.24± 11.85c	9.57± 0.16c	ND	457.69± 8.49
GK	ND	ND	ND	523.64± 28.44a	ND	ND	ND	21.81± 0.88b	2.81± 0.14c	268.65± 2.57b	38.46± 0.35b	1.42± 0.64a	856.80± 29.50
GL	11.44± 1.99a	ND	ND	366.23± 30.05b	4.72± 0.50c	ND	ND	25.10± 0.86a	2.02± 0.06c	405.23± 8.66a	41.66± 0.88a	ND	856.41± 24.12
GC	ND	ND	ND	0.83± 0.04e	ND	ND	ND	ND	ND	0.14± 0.01f	0.03±0.00f	ND	0.99± 0.04

[a]Different letters represent significant differences (p <0.05) between means determined by a one-way analysis of variance followed by a Duncan multiple-range test. Each value represents the mean ± standard deviation (n =3). [b]Viola, violaxanthin; Anthera, antheraxanthin; α-Crypto, α-cryptoxanthin; β-Crypto, β-cryptoxanthin; 13*Z*-β-Caro, 13*Z*-β-carotene; *E*-β-Caro, *E*-β-carotene; 9*Z*-β-Caro, 9*Z*-β-carotene. [c]YP, yellow paprika; OP, orange paprika; RP, red paprika; GK, green kale; GL, green lettuce; GC, green cabbage. [d]ND = not detectable.

Table 2 Correlations among carotenoid contents of paprika

	Viola[a]	Anthera	Capsanthin	Lutein	Zeaxanthin	α-Crypto	β-Crypto	13Z-β-Caro	α-Carotene	E-β-Caro	9Z-β-Caro	Lycopene
Viola	1.000											
Anthera	-0.068	1.000										
Capsanthin	0.062	0.987*	1.000									
Lutein	-0.617	-0.726	-0.813	1.000								
Zeaxanthin	-0.918	-0.253	-0.381	0.847	1.000							
α-Crypto	-0.885	-0.339	-0.463	0.892	0.995*	1.000						
β-Crypto	-0.884	0.463	0.339	0.269	0.739	0.674	1.000					
13Z-β-Caro	-0.292	0.949*	0.902	-0.523	-0.004	-0.089	0.664	1.000				
α-Carotene	-0.794	-0.517	-0.635	0.961*	0.947	0.970*	0.501	-0.292	1.000			
E-β-Caro	-0.269	0.973*	0.931	-0.563	-0.045	-0.132	0.637	0.991*	-0.328	1.000		
9Z-β-Caro	-0.200	0.989*	0.963*	-0.634	-0.130	-0.219	0.569	0.973*	-0.410	0.992*	1.000	
Lycopene	-0.838	-0.355	-0.488	0.881	0.954*	0.967*	0.614	-0.134	0.970*	-0.164	-0.253	1.000

[a]Viola, violaxanthin; Anthera, antheraxanthin; α-Crypto, α-cryptoxanthin; β-Crypto, β-cryptoxanthin; 13Z-β-Caro, 13Z-β-carotene; E-β-Caro, E-β-carotene; 9Z-β-Caro, 9Z-β-carotene. *Significant at 0.0001 probability level.

Thus, additional information can be obtained from functional genomic studies. In carotenoid biosynthesis, β-cryptoxanthin and α-carotene convert into zeaxanthin and letein, respectively. In the results of Pearson's correlation analysis conducted to examine the relationship between carotenoid levels (Table 2), significant positive relationships were apparent between capsanthin and antheraxanthin (r =0.9870, p <0.0001), zeaxanthin and α-cryptoxanthin (r = 0.9951, p <0.0001), as well as lutein and α-carotene (r =0.9612, p <0.0001). Therefore, these results indicate that the distribution of the carotenoids should differ based on the carotenoid biosynthesis pathway in the plants.

In conclusion, the carotenoids in cabbage, kale, lettuce, and various colored paprikas marketed in Korea were identified by HPLC with a C30 reverse-phase stationary matrix. The major contributors to the total carotenoid content of the vegetables were lutein and all-trans-β-carotene. In applying the profiling concept, it is crucial to perform an unbiased multi-targeted metabolite analysis in order to precisely elucidate the biochemical functions in plant metabolism. The application of this method to screening the carotenoid content of several fruits and vegetables from Korea and the depiction of the carotenoid biosynthesis pathways have been described to illustrate the versatility of the procedure.

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